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13. ABSTRACT (Maximum 200 words)

The relative inhibitory potency (RIP) of an organophosphorus (OP) inhibitor against acetylcholinesterase(AChE) versus neuropathy target esterase (NTE) is [ki(AChE)/ki(NTE)], where ki is the bimolecular rate constant of inhibition. RIPs>1 correlate with the inability of ageable OP inhibitors or their parent compounds to produce OP–induced delayed neurotoxicity (OPIDN) at doses <LD50. The RIP for chlorpyrifos oxon (CPO) is >>1 for enzymes from hen brain homogenate, and the parent compound, chlorpyrifos (CPS), cannot produce OPIDN in hens at sublethal doses. This study was done to test the hypothesis that the RIP for chlorpyrifos methyl oxon (CPMO), is >>1 and >RIP for CPO. Mipafox (MIP) was included for comparison. Hen brain microsomes were used as the enzyme source, and ki (mean ± SE, µM^ 1min^- 1) determined for AChE and NTE (n = 3, 4 experiments, respectively). ki values for CPO, CPMO, and MIP against AChE were 17.8±0.3, 10.9±0.1, and 0.00429±0.00001, respectively, and for NTE were 0.0993±0.0049, 0.0582±0.0013, and 0.00498±0.00006, respectively. Corresponding RIPs for CPO, CPMO, and MIP were 179±9, 187±4, and 0.861±0.011, respectively. Thus, RIPs for CPO and CPMO are comparable, markedly different from that for MIP, and >>1, indicating that CPS methyl, like CPS, could not cause OPIDN at sublethal doses.

14. SUBJECT TERMS

Acetylcholinesterase (AChE), Delayed neurotoxicity (OPIDN), Neuropathy Target Esterase (NTE), Organophosphorus compounds (OPs), Quantitative Structure-Activity Relationships (QSAR), Relative inhibitory potency (RIP).

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RELATIVE INHIBITORY POTENCIES OF CHLORPYRIFOS OXON, CHLORPYRIFOS METHYL OXON, AND MIPAFOX FOR ACETYLCHOLINESTERASE VERSUS NEUROPATHY TARGET ESTERASE

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The relative inhibitory potency (RIP) of an organophosphorus (OP) inhibitor against acetylcholinesterase (AChE) versus neuropathy target esterase (NTE) may be defined as the ratio [k_i(AChE)/k_i(NTE)], where k_i is the bimolecular rate constant of inhibition for a given inhibitor against each enzyme. RIPs greater than 1 correlate with the inability of ageable OP inhibitors or their parent compounds to produce OP compound–induced delayed neurotoxicity (OPIDN) at doses below the LD50. The RIP for chlorpyrifos oxon (CPO) is >>1 for enzymes from hen brain homogenate, and the parent compound, chlorpyrifos (CPS), cannot produce OPIDN in hens at sublethal doses. This study was carried out to test the hypothesis that the RIP for the methyl homologue of CPO, chlorpyrifos methyl oxon (CPMO), is >>1 and greater than the RIP for CPO. Mipafox (MIP), an OP compound known to produce OPIDN, was included for comparison. Hen brain microsomes were used as the enzyme source, and k_i values (mean ± SE, $\mu M^{-1}min^{-1}$) were determined for AChE and NTE (n = 3 and 4 separate experiments, respectively). The k_i values for CPO, CPMO, and MIP against AChE were 17.8 ± 0.3, 10.9 ± 0.1, and 0.00429 ± 0.00001, respectively, and for NTE were 0.0993 ± 0.0049, 0.0582 ± 0.0013, and 0.00498 ± 0.00006, respectively. Corresponding RIPs for CPO, CPMO, and MIP were 179 ± 9, 187 ± 4, and 0.861 ± 0.011, respectively. The results demonstrate that RIPs for CPO and CPMO are comparable, markedly different from that for MIP, and >>1, indicating that CPS methyl, like CPS, could not cause OPIDN at sublethal doses.

The phosphorothionate insecticides *O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothionate (chlorpyrifos; CPS) and *O*,*O*-dimethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothionate (chlorpyrifos methyl; CPMS), require metabolic activation via oxidative desulfuration to their respective oxon forms, *O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphate (chlorpyrifos oxon; CPO) and *O*,*O*-dimethyl *O*-3,5,6-trichloro-2-pyridyl phosphate (chlorpyrifos methyl oxon; CPMO), in order

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to be effective inhibitors of acetylcholinesterase (AChE) or other serine esterases (Chambers & Chambers, 1989). The intended mode of action of these and other organophosphorus (OP) insecticides is to bring about acute cholinergic toxicity by inhibiting AChE in nerve endings (Mileson et al., 1998; Moretto, 1998; Pope, 1999). An untoward effect of some OP compounds is to produce OP compound–induced delayed neurotoxicity (OPIDN) through the inhibition and aging of neuropathy target esterase (NTE) in neural tissue (Glynn, 1999). Aging of inhibited NTE involves postinhibitory loss of a labile moiety from the inhibitor, leaving a negatively charged phosphyl adduct covalently attached to the active site serine (Richardson, 1998). Ageable inhibitors include molecules whose postinhibitory structures before aging have at least one -OR group (e.g., phosphates and phosphonates) or -NHR group (e.g., phosphoramidates), where R=alkyl or aryl (Davis et al., 1985).

Previous work has shown that the relative inhibitory potency (RIP) in vitro of the active oxon form of an ageable organophosphorus (OP) compound against AChE versus NTE correlates with the ability of the parent compound to produce OPIDN in vivo (Lotti & Johnson, 1978; Makhaeva et al., 1998). The inhibitory potency of an OP oxon against AChE or NTE may be assessed by measuring its k_i value for each enzyme, and its RIP for these targets may be determined by calculating the ratio $[k_i(\text{AChE})/k_i(\text{NTE})]$ (Richardson, 1992). When pseudo–first-order kinetics are obtained and the apparent first-order rate constants are linearly related to inhibitor concentration, it is valid to calculate the fixed-time I_{50} value from the relationship $I_{50}=(\ln 2)/(k_i \times t)$, where I_{50} is the inhibitor concentration that produces 50% inhibition of enzyme activity under specified conditions, and t is the time of preincubation of the inhibitor with the enzyme (Aldridge & Reiner, 1972). Moreover, under these kinetic conditions, experimentally determined I_{50} values may be used to calculate RIPs (Richardson et al., 1993). When the RIP, calculated either as the $[k_i(\text{AChE})/k_i(\text{NTE})]$ ratio or the $[I_{50}(\text{NTE})/I_{50}(\text{AChE})]$ ratio, is greater than 1, the inhibitor has a greater inhibitory potency against AChE than NTE, and doses of the parent compound greater than the LD50 are required to produce OPIDN (Lotti & Johnson, 1978; Makhaeva et al., 1998; Richardson, 1992).

Based on fixed-time I_{50} values under conditions of nonlinear kinetics in hen brain homogenates (20 min at 37 °C), the RIP for CPO has been determined to be 25 (Capodicasa et al., 1991). Using kinetic determinations of k_i values obtained from linear kinetics, the corresponding RIP was found to be 107 (Richardson et al., 1993). In either case, it is clear that the RIP of CPO is $>>1$, indicating that CPS could not produce OPIDN at sublethal doses, consistent with experimental results in animals (Capodicasa et al., 1991; Richardson et al., 1993) and clinical data on humans (Lotti et al., 1986; Moretto & Lotti, 1998).

In contrast to the substantial amount of information about the safety of CPS with respect to delayed neurotoxicity (Richardson, 1995), data are lacking concerning the propensity for CPMS, the methyl homologue of CPS, to produce OPIDN. Nevertheless, as had been observed for CPO in previous studies using hen brain homogenates (Capodicasa et al., 1991; Richardson et al., 1993),

it may be hypothesized that the RIP for its methyl homologue, CPMO, would also be >>1 and greater than the RIP for CPO. This contention arises from structure-activity studies showing that decreasing the carbon chain length of alkyl substituents attached to phosphorus tends to increase the RIP and decrease the neuropathic potential for a given homologous series of dialkyl phosphates (Makhaeva et al., 1998; Richardson, 1992). If the RIP data for CPMO and CPO were to support the prediction from structure-activity considerations, then this would indicate that the potential for CPMS to produce OPIDN would be even lower than that of CPS, which is already known to be incapable of producing OPIDN at sublethal doses (Capodicasa et al., 1991; Moretto & Lotti, 1998; Richardson, 1995; Richardson et al., 1993).

In order to provide a basis for assessing the delayed neuropathic potential of CPMS relative to CPS, the present study was carried out to test the hypothesis that the RIP for CPMO is >>1 and greater than the RIP for CPO. Inhibitory potencies were determined by measuring k_i values for CPO, CPMO, and *N*,*N*′-diisopropylphosphorodiamidic fluoride (mipafox; MIP) against hen brain microsomal AChE and NTE, and the RIP values were calculated. MIP, an OP compound known to produce OPIDN (Davis et al., 1985), was included for contrast. Previous work indicated that MIP would have comparable inhibitory potencies for AChE and NTE, and would be far less potent against either enzyme than CPO or CPMO (Ehrich et al., 1997; Kropp & Richardson, 2002; Lotti & Johnson, 1978). Adult hen brain was used as the enzyme source in the present study, because assays of brain AChE and NTE from this species are required by domestic (U.S. EPA, 1998) and international (OECD, 1995a, 1995b) regulatory agencies for neurotoxicity testing of OP compounds. The current study employed hen brain microsomes in order to achieve more uniform suspensions of these membrane-bound enzymes than would be possible in crude homogenates (Jianmongkol et al., 1996; Richardson et al., 1979) and to reduce concentrations of extrinsic components (Mortensen et al., 1998) that may be present in the larger particulates and/or the soluble fraction contained in homogenates of whole brain.

MATERIALS AND METHODS

Preparation of Hen Brain Microsomal Membranes

Brains from single-comb white Leghorn hens 20 to 24 mo of age (adult female of *Gallus domesticus*) were obtained from the Department of Animal Sciences, Michigan State University (East Lansing). Following termination of hens by cervical dislocation, brains were quickly removed, submerged in ice-cold isotonic saline, stripped of meninges and superficial blood vessels, blotted and weighed, frozen on dry ice, and shipped to the laboratory overnight on dry ice. Using a method similar to that of Richardson et al. (1979) as modified by Jianmongkol et al. (1996), a microsomal membrane suspension was prepared from the pooled homogenates of three brains and used as the source of AChE

and NTE. Briefly, following thawing in an ice bath, a 10% (w/v) homogenate of each brain was made in ice-cold 0.32 *M* sucrose using 15 strokes of a glass-Teflon homogenizer with 0.25 mm clearance operating at 1325 rev/min. Pooled homogenate was centrifuged at 14,400×g for 20 min at 4 °C (all values of g refer to the g-force at the average radius of the tube). The supernatant was centrifuged at 104,000×g for 60 min at 4 °C to yield a microsomal pellet. To make a stock enzyme suspension, the microsomal pellet was resuspended to 20% (w/v) in 0.32 *M* sucrose/0.20 m*M* ethylenediamine tetraacetic acid (EDTA), aliquoted, and stored at −80 °C until use.

AChE Inhibition Kinetics

Stock enzyme suspensions were diluted with phosphate buffer (0.1 *M* sodium phosphate, pH 7.6) to achieve a control rate of substrate hydrolysis of approximately 0.05 absorbance unit/min. Enzyme kinetics were performed in phosphate buffer at 37 °C using a modification of the method of Ellman et al. (1961) to monitor AChE activity. CPO (lot AGR203674; identity by infrared [IR]; purity 96% by gas chromatography [GC]; Dow AgroSciences, Indianapolis, IN) or CPMO (lot TSN102205; identity by nuclear magnetic resonance [NMR], IR, and mass spectrometry [MS]; purity 98% by LC and GC; Dow AgroSciences) was dissolved in high-performance liquid chromatography (HPLC)-grade acetone and diluted in phosphate buffer. Final concentrations of acetone were <0.1% (v/v) and did not affect enzyme activity in the absence of OP inhibitors. MIP (identity by NMR, purity ≥98% by elemental analysis, mp 60.5–62.0 °C; ChemSyn Laboratories, Lenexa, KS) was dissolved in 50 m*M* Tris-citrate buffer, pH 6.0 (Johnson, 1977), and diluted in phosphate buffer. Enzyme and inhibitor were preincubated together for various timed periods up to a maximum of 12 min at 37 °C. At the end of each preincubation interval, substrate solution containing acetylthiocholine (final concentration 1.0 m*M*) and 5,5′-dithiobis-(2-nitrobenzoic acid) (final concentration 0.32 m*M*) (both from Sigma Chemical Co., St. Louis, MO) were added. Residual enzyme activity was determined by measuring the change in absorbance at 412 nm over a 1.5-min period at 37 °C using a SPECTRAmax 340 microplate reader (Molecular Devices Corp., Sunnyvale, CA).

NTE Inhibition Kinetics

Stock enzyme suspensions were diluted with Tris buffer (50 m*M* Tris-HCl/0.20 m*M* EDTA, pH 8.0, at 25 °C) to yield a control rate of substrate hydrolysis in the range of 0.01 to 0.05 absorbance unit/min. Enzyme kinetics were performed at 37 °C using a modification of the method of Kayyali et al. (1991) based on the original method of Johnson (1977) to measure residual NTE activity. A set of paired aliquots of enzyme were preincubated at 37 °C for 20 min with diethyl 4-nitrophenyl phosphate (paraoxon; Sigma Chemical Co., St. Louis, MO) (final concentration 100 μ*M*) plus Tris buffer or paraoxon (final concentration 100 μ*M*) plus MIP (final concentration 50 μ*M*). At the end of the initial 20-min preincubation period, inhibitor (CPO or CPMO dissolved in HPLC-

grade acetone and diluted in Tris buffer, or MIP dissolved in 50 m*M* Tris-citrate buffer, pH 6.0, and diluted in Tris buffer) was added and carried through a second preincubation interval for various timed periods (up to a maximum of 9 min for CPMO or 16 min for CPO and MIP) at 37 °C. Final acetone concentrations for preincubations with CPO or CPMO were <0.1% (v/v) and did not affect enzyme activity in the absence of these inhibitors. Identity and purity information on the inhibitors CPO, CPMO, and MIP are given in the section on AChE inhibition kinetics. At the end of each of the second preincubation periods, substrate (phenyl valerate; Oryza Laboratories, Chelmsford, MA) was added (final nominal concentration 2.65 m*M*) and incubation was carried out for a timed interval of 30 to 60 min. Enzyme reaction was stopped by the addition of 4-aminoantipyrine in aqueous sodium dodecyl sulfate (final concentrations 0.41 m*M* and 3.2 mg/ml, respectively). Color was then developed by adding potassium ferricyanide (final concentration 0.06% w/v) and absorbance was read at 486 nm using a SPECTRAmax 340 microplate reader (Molecular Devices Corp., Sunnyvale, CA).

Determination of k_i and I_{50} Values

The k_i values for inhibition of AChE or NTE by each inhibitor were measured according to established methods (Aldridge & Reiner, 1972; Richardson 1992). Each inhibitory reaction was carried out with $[I] > 10\times[\text{Enzyme}]$ and $[I] < K_a$, so that the k_i could be determined using pseudo–first-order kinetics; $[I]$ is the concentration of inhibitor (CPO, CPMO, or MIP) and K_a is the affinity constant for the Michaelis-type complex between the enzyme and inhibitor. Primary kinetic plots of ln (percent activity remaining) versus time (t) were generated for various values of $[I]$. The slopes of these plots determined by linear regression yielded apparent first-order rate constants, k', for each value of $[I]$. The secondary kinetic plot of $-k'$ versus $[I]$ was then generated and its slope was determined by linear regression to yield the k_i. For each experiment, 4 time points and 5 to 6 inhibitor concentrations (in duplicate) were used, and 3 or 4 separate experiments were done to determine the mean ± standard error (SE) for the k_i value for each inhibitor against hen brain AChE or NTE, respectively. For comparison, the corresponding fixed-time ($t = 20$ min) I_{50} values were also calculated from the measured k_i values using the relationship $I_{50} = (\ln 2)/(k_i \times t)$ (Aldridge & Reiner, 1972; Richardson, 1992).

Calculation of [k_i(AChE)/k_i(NTE)] Ratios (RIP Values)

The mean [k_i(AChE)/k_i(NTE)] ratios (RIP values) for CPO, CPMO, and MIP were calculated from the means of the respective measured k_i values. The SE for each ratio was calculated based on the propagation of uncertainty for quotients (Harris, 1991).

Statistical Analyses

All data are presented as means ± SE. Linear regression was used to obtain k_i values from primary and secondary kinetic plots. Significance of differences between means of k_i or I_{50} values for different inhibitors for a given enzyme

was assessed by one-way analysis of variance (ANOVA) ($\alpha = .05$) using the Tukey post hoc test for pairwise comparisons. Significance of differences between means of k_i or I_{50} values across enzymes for each inhibitor was determined by two-way ANOVA ($\alpha = .05$) using the Bonferroni posttest for pairwise comparisons. Differences among RIP values were evaluated by examining overlap of associated SE values and the 95% confidence intervals. Determination of significant differences of RIP values from a constant value of 1 was done by transforming the k_i values for a given inhibitor against each enzyme to their respective log k_i values and examining the significance of the difference between the mean log k_i values using two-way ANOVA ($\alpha = .05$) with the Bonferroni posttest. A log transformation was done, because the distribution of values of ratios is inherently asymmetric (Motulsky, 1995). Given that [log k_i (AChE) – log k_i (NTE)] = log[k_i (AChE)/k_i (NTE)], if the difference [log k_i(AChE) – log k_i(NTE)] is significant (i.e., the difference $\neq 0$), then the ratio [k_i(AChE)/k_i(NTE)] $\neq 1$, because antilog (0) = 1. Kinetic plots and statistical calculations were done using GraphPad Prism version 3.02 for Windows, GraphPad Software, Inc. (San Diego, CA).

RESULTS

Pseudo–first-order kinetic behavior was observed in all primary inhibition plots for all inhibitors against AChE and NTE. Moreover, the slopes of the primary plots were linearly related to inhibitor concentration. This kinetic behavior is exemplified in Figures 1 and 2, which show representative primary plots and their associated secondary plots for CPMO against AChE and NTE, respectively. Thus, it was possible to determine reliable k_i values from which I_{50} values could also be calculated (Aldridge & Reiner, 1972; Richardson, 1992).

Table 1 shows the measured k_i values and calculated 20-min I_{50} values for CPO, CPMO, and MIP against hen brain microsomal AChE and NTE. The calculated RIP values, that is, the [k_i(AChE)/k_i(NTE)] ratios, are also shown. Note that the RIP values based on k_i ratios are equivalent to the reciprocal ratios based on calculated I_{50} values, that is, [k_i(AChE)/k_i(NTE)] = [I_{50}(NTE)/I_{50}(AChE)].

Inspection of Table 1 reveals that the order of potency of the three inhibitors against either AChE or NTE was found to be CPO > CPMO >> MIP. It is apparent that the k_i values (or the corresponding I_{50} values) of each inhibitor against a given enzyme were significantly different from each other (one-way ANOVA with Tukey posttest, $p < .05$). The inhibitor constants for a given inhibitor were also each significantly different for AChE versus NTE (two-way ANOVA with Bonferroni posttest, $p < .05$). The RIP values for CPO and CPMO were 208- and 217-fold greater, respectively, than the value for MIP. It can be seen that the RIP values for CPO and CPMO were not different from each other, because their respective mean ± SE values overlapped. At the same time, the RIP values for CPO and CPMO differed from that for MIP, because the 95% confidence intervals for CPO and CPMO did not overlap with that for

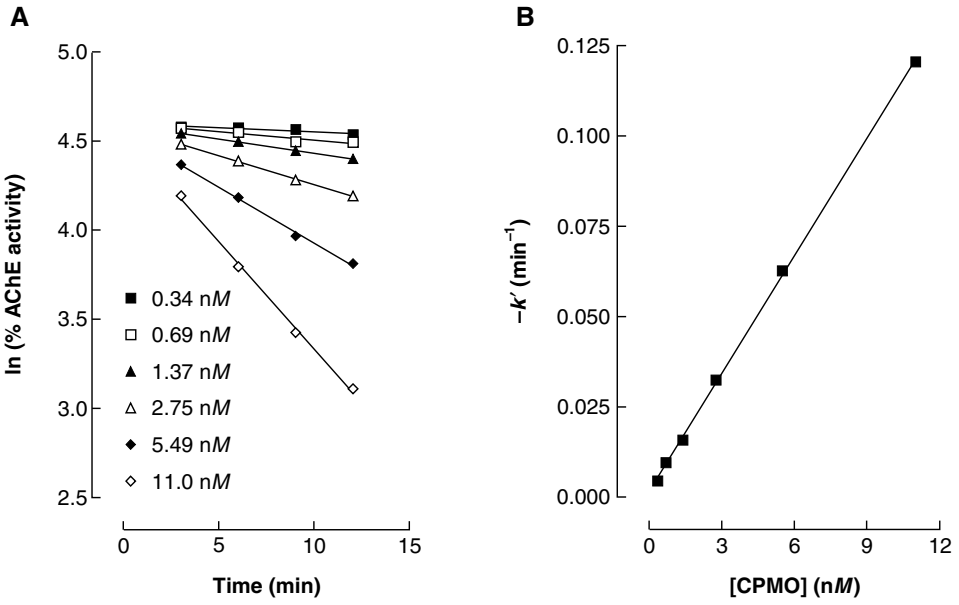

FIGURE 1. (A) Representative primary plots of the time course of inhibition of AChE by different concentrations of CPMO. The slope of each line is the apparent first-order rate constant at a given inhibitor concentration, $-k'$ (min^{-1}). (B) Secondary plot of $-k'$ versus [CPMO] (n*M*); the slope of the line is the bimolecular rate constant of inhibition (k_i) for CPMO against AChE.

MIP (the 95% confidence intervals for the RIP values were as follows: 153 to 219 for CPO; 175 to 199 for CPMO; and 0.829 to 0.896 for MIP). Finally, by transforming k_i values to their respective log k_i values and examining the significance of differences between AChE and NTE for each inhibitor (two-way ANOVA with Bonferroni posttest, $p < .05$), it was determined that the difference [log k_i(AChE) – log k_i(NTE)] was significantly different from zero for each inhibitor. Therefore, the log of each ratio, log [k_i(AChE)/k_i(NTE)], was significantly different from 1. This statistical result, in combination with an examination of the values given in Table 1, demonstrates that the RIP values for CPO and CPMO were significantly greater than 1 and that the RIP value for MIP was significantly less than 1.

DISCUSSION AND CONCLUSION

The first part of the hypothesis of the present study was that the RIP of CPO and CPMO as assessed by measurement of the [k_i(AChE)/k_i(NTE)] ratio would be substantially greater than 1. Indeed, the values obtained for CPO and CPMO were 179±9 and 187±4, respectively. These values are clearly >>1 and were found to be significantly so, supporting this part of the hypothesis. The results for CPO and CPMO were in sharp contrast to those obtained

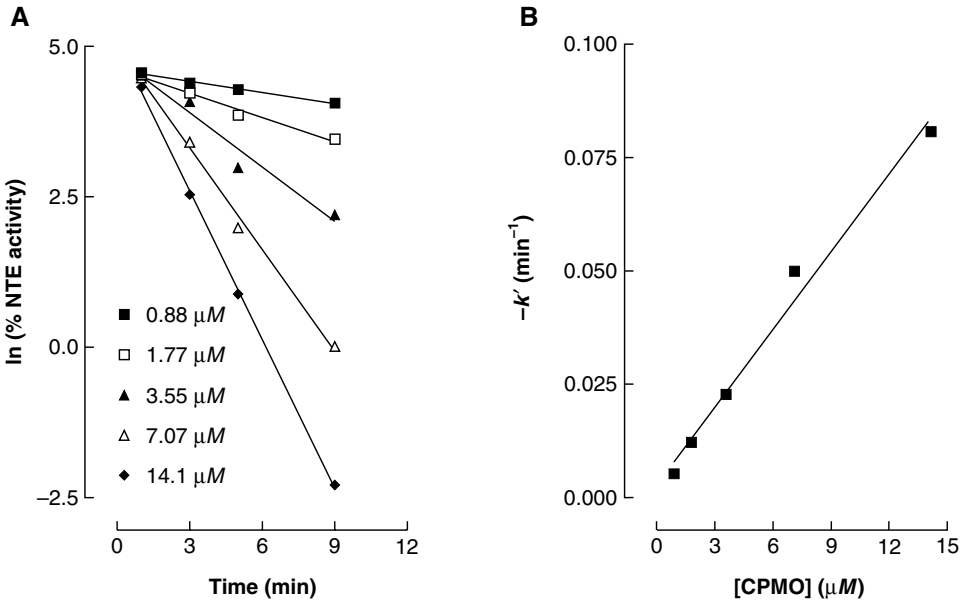

FIGURE 2. (A) Representative primary plots of the time course of inhibition of NTE by different concentrations of CPMO. The slope of each line is the apparent first-order rate constant at a given inhibitor concentration, $-k'$ (min^{-1}). (B) Secondary plot of $-k'$ versus [CPMO] (μM); the slope of the line is the bimolecular rate constant of inhibition (k_i) for CPMO against NTE.

for the reference compound, MIP. Not only were the corresponding individual k_i values markedly different, with MIP showing substantially lower potency than either CPO or CPMO for both enzymes, but the RIP for MIP was significantly less than 1 (0.861 ± 0.011) and far below the values obtained for CPO and CPMO (208- and 217-fold, respectively).

The second part of the hypothesis for this project, based on structure-activity considerations from other compounds (Makhaeva et al., 1998; Richardson, 1992), was that the RIP for CPMO could be somewhat larger than that for CPO. Although the mean value of the RIP for CPMO was larger than that for CPO, the difference between them was obviously not statistically significant given the overlap in their mean ± SE values. Therefore, the second hypothesis was not upheld by the data. Nevertheless, it was apparent that CPMO favors inhibition of AChE over NTE to at least the same degree as that exhibited by CPO.

The data presented in the current study are likely to be the best reflection of the true absolute and relative inhibitory potencies of CPO against hen brain AChE and NTE obtained to date. There are three reasons for this statement. First, the present work employed kinetic methods that are thought to be more valid than fixed-time methods for determinations of inhibitory potencies of OP compounds against esterases (Clothier et al., 1981; Fukuto, 1990; Richardson,

TABLE 1. Inhibitor Constants and RIP Values for CPO, CPMO, and MIP Against Hen Brain Microsomal AChE and NTE

	k_i (μM^{-1} min^{-1})[a]		20-min I_{50} (μM)[b]		
Inhibitor	AChE	NTE	AChE	NTE	RIP[c]
CPO	17.8 ± 0.3	0.0993 ± 0.0049	0.00195 ± 0.00003	0.349 ± 0.017	179 ± 9
CPMO	10.9 ± 0.1	0.0582 ± 0.0013	0.00318 ± 0.00003	0.595 ± 0.013	187 ± 4
MIP	0.00429 ± 0.00001	0.00498 ± 0.00006	8.08 ± 0.02	6.96 ± 0.08	0.861 ± 0.011

Note. Values are means ± SE ($n = 3$ for AChE and $n = 4$ for NTE, where n = number of separate experiments). CPO, chlorpyrifos oxon; CPMO, chlorpyrifos methyl oxon; MIP, mipafox; AChE, acetylcholinesterase; NTE, neuropathy target esterase.

[a] Bimolecular rate constant of inhibition. The k_i values for each inhibitor against a given enzyme are significantly different from each other (one-way ANOVA, Tukey posttest, $p < .05$). The k_i values for a given inhibitor are also each significantly different for AChE versus NTE (two-way ANOVA, Bonferroni posttest, $p < .05$).

[b] Concentration of inhibitor producing 50% inhibition of enzymatic activity after a preincubation interval (t) of 20 min with a given inhibitor. Calculated from the k_i using the relationship $I_{50} = 0.693/(k_i \times t)$. The I_{50} values of each inhibitor against a given enzyme are significantly different from each other (one-way ANOVA with Tukey post test, $p < .05$). The I_{50} values for a given inhibitor are also each significantly different for AChE versus NTE (two-way ANOVA, Bonferroni posttest, $p < .05$).

[c] RIP, relative inhibitory potency = $[k_i(\text{AChE})/k_i(\text{NTE})] = [I_{50}(\text{NTE})/I_{50}(\text{AChE})]$. Mean RIP values for each inhibitor were calculated from the means of the respective measured k_i or calculated I_{50} values. The SE for each ratio was calculated based on the propagation of uncertainty for quotients (Harris, 1991). RIP values for CPO and CPMO are not significantly different from each other (overlapping mean ± SE). RIP values for CPO and CPMO are significantly different from that for MIP (95% confidence intervals [CI] for CPO and CPMO do not overlap with that for MIP (95% CI for RIP values = 153 to 219 for CPO; 175 to 199 for CPMO; and 0.829 to 0.896 for MIP). RIP values for CPO and CPMO are significantly greater than 1 and that for MIP is significantly less than 1 (two-way ANOVA, Bonferroni posttest, $p < .05$ on differences between log-transformed k_i values between AChE and NTE for each inhibitor; see the Materials and Methods and the Results sections for details of this analysis).

1992; Richardson et al., 1993). Second, the kinetic measurements of the present work used more time points, inhibitor concentrations per experiment, and separate experiments than were done in previous determinations of k_i values for CPO against hen brain AChE and NTE (Richardson et al., 1993). Third, the interference of extrinsic factors that may be present in whole tissue homogenates (Mortensen et al., 1998) was minimized by using microsomal preparations in the current investigation.

As the present study was the first to report the inhibitory potency of CPMO against either AChE or NTE in hen brain, no direct parallels could be drawn with previously published values. Nevertheless, it may be instructive to contrast the results reported here with published work on the apparent inhibitory potency of CPMO against AChE from other systems.

CPO was determined here to be only 1.6 times as potent as CPMO against hen brain microsomal AChE as assessed kinetically at 37 °C. Sultatos et al. (1982), however, using fixed-time (1-h) I_{50} values at 27 °C found that CPO was about

480 times more potent than CPMO against AChE from bovine erythrocytes or mouse brain homogenates. One source of the major variation in the results for CPMO potency obtained via kinetics in the present study versus fixed-time incubation in the work of Sultatos et al. (1982) is likely to be the intrinsically high reactivation rate of AChE inhibited by dimethyl phosphates. It is known that AChE phosphorylated with dimethyl phosphates reactivates relatively rapidly ($t_{1/2} \approx 1$ h) as compared to AChE inhibited by diethyl phosphates, which reactivates relatively slowly ($t_{1/2} \approx 60$ h) (Aldridge & Reiner, 1972; Clothier et al., 1981). Hence, during the 1-h preincubation period used by Sultatos et al. (1982), extensive reactivation of AChE would be taking place during inhibition with the dimethyl phosphate, CPMO. Indeed, in preliminary experiments carried out for the current work, curvilinear primary kinetic plots consistent with reactivation (Aldridge & Reiner, 1972; Richardson, 1992) were obtained with CPMO, but not CPO or MIP, when preincubation periods were longer than 12 min for AChE or 9 min for NTE (data not shown). Preincubation times were therefore restricted to no more than 12 min for AChE or 9 min for NTE in experiments with CPMO. Nevertheless, reactivation alone would not be sufficient to explain the discrepancy between the kinetically determined and fixed-time potencies, and the relative stabilities of the inhibitors themselves should be considered (L. Sultatos, personal communication). For example, it is known that CPMS is hydrolyzed over 100 times faster than CPS, and the hydrolysis rate of OP oxons is typically 2 to 20 times greater than their corresponding phosphorothionates (Eto, 1974). In any event, the k_i values reported here reflect maximal initial rates of inhibition obtained under conditions of pseudo–first-order kinetics, as has been recommended for doing consistent comparisons of inhibitory potency (Clothier et al., 1981; Fukuto, 1990; Richardson, 1992; Richardson et al., 1993).

A k_i value of 24.0 $\mu M^{-1}\text{min}^{-1}$ (corresponding to a 20-min I_{50} of 1.4 n*M*) was reported in a study employing room-temperature incubation of CPMO against a purified insect AChE (Gao et al., 1998). This kinetically determined value from a purified insect AChE is within a factor of 2.2 of the k_i of 10.9 $\mu M^{-1}\text{min}^{-1}$ of CPMO obtained in the current study. It is noteworthy that the kinetic procedure of Gao et al. (1998) used a relatively short incubation time with inhibitor of only 2 min and linear kinetic behavior was obtained. The relatively close agreement between the current kinetically determined result and that of Gao et al. (1998) further supports the contention stated earlier that the discrepant results of Sultatos et al. (1982) may have arisen from the rapid reactivation of dimethyl phosphoryl–AChE and/or hydrolysis of the inhibitor.

With respect to measurements of the inhibitory potency of CPO, Amitai et al. (1998) measured the k_i of this compound against different sources of purified AChE at 25 °C. Their values for fetal bovine serum, human erythrocyte, *Torpedo californica*, recombinant mouse, and recombinant human AChE were 2.2, 3.8, 8.0, 5.1, and 9.3 $\mu M^{-1}\text{min}^{-1}$, respectively. Adjusting the k_i obtained in the present work to account for the temperature difference between the two studies (Aldridge & Reiner, 1972) yields a value of 7.4 $\mu M^{-1}\text{min}^{-1}$ for hen

brain microsomal AChE, which is within the range obtained by Amitai et al. (1998).

Capodicasa et al. (1991) investigated the time course of inhibition of hen brain AChE and NTE by CPO under conditions that gave nonlinear kinetics and determined 20-min I_{50} values of 6 and 150 n*M* for AChE and NTE, respectively. Their RIP was therefore 25. Another previous study found that the k_i values for CPO against AChE and NTE from hen brain homogenates were 15.5 and 0.145 $\mu M^{-1}min^{-1}$, respectively (Richardson et al., 1993), yielding an RIP of 107. The present study using kinetic methods and hen brain microsomes yielded a corresponding RIP of 179.

The current study was the first to report a k_i determination for MIP against AChE and NTE as well as the first to provide an assessment of the inhibitory potency for this compound against hen brain microsomal esterases. Previous work found that the 20-min I_{50} for MIP against NTE in hen brain homogenates at 37 °C was 7.8 μM (Dudek & Richardson, 1982) and 7.4, 6.6, and 8.1 μM when solubilized with Triton X-100, sodium cholate, or sodium cholate plus asolectin, respectively (Davis & Richardson, 1987). Using a partially purified hen brain homogenate, Novak and Padilla (1986) reported the 20-min I_{50} for MIP at 37 °C to be 7.3 μM and Lotti and Johnson (1978) reported a value of 7 μM. All these I_{50} values for MIP against NTE are in excellent agreement with the value of 6.96 μM determined for hen brain microsomal NTE in the present study. Of particular interest for the present study, Ehrich et al. (1997) found RIP values for MIP and CPO in mouse neuroblastoma cells to be 2.6 and 87, respectively. In human neuroblastoma cells, these authors found RIP values for MIP and CPO of 1.1 and 200, respectively. These results are in good agreement with the RIP values found in the current study of 0.86 and 179 for MIP and CPO, respectively.

There appears to be only a single previous investigation of the inhibitory potency of MIP against hen brain AChE. Lotti and Johnson (1978) reported an I_{50} of MIP against AChE of 41 μM. This value is about fivefold greater than the one obtained in the current report. However, the previous study reported mean values obtained from only two experiments that employed different conditions from those of the present work. Moreover, the previous study used a temperature conversion factor to arrive at the published number for AChE, and no indications of variance were given. The RIP value from the Lotti and Johnson (1978) study for MIP was 0.17, as compared to 0.861 obtained in the current work. Although these RIP values differ by a factor of 5, they are both less than 1, and the conclusion reached in both cases is that the relative inhibitory behavior of MIP toward NTE and AChE is markedly different from that of CPO and CPMO.

Before the current study, there were no data available for estimating the RIP for CPMO. These data now exist and enable the calculation of a RIP value of 187. This number has been shown to be significantly greater than 1 and not different statistically from the RIP value of 179 for CPO. Given that RIP values of this magnitude correlate with the inability of the inhibitor itself or its

parent compound to cause OPIDN at less than lethal doses (Capodicasa et al., 1991; Ehrich et al., 1997; Lotti & Johnson, 1978; Richardson, 1992, 1995; Richardson et al., 1993), an important conclusion that may be drawn from the results of the present study is that CPMS, like its analogue CPS, cannot produce OPIDN at sublethal doses.

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